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(54) Title: LIGHT-REGULATED PROMOTERS FOR PRODUCTION OF HETEROLOGOUS PROTEINS IN FILAMENTOUS FUNGI		
(57) Abstract		
<p>The al-3 and related promoters can be used to provide light-regulated recombinant production of heterologous proteins in filamentous fungi. Expression systems utilizing these promoters can be placed in vectors which also optionally contain selectable marker means.</p>		

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LIGHT-REGULATED PROMOTERS FOR PRODUCTION OF HETEROLOGOUS
PROTEINS IN FILAMENTOUS FUNGI

Technical Field

The invention relates to recombinant production of
5 heterologous, especially eucaryotic, proteins in filamentous
fungal hosts. In particular, it relates to expression
constructs which utilize light-regulated fungal promoters.

Background Art

It has been known for many years that exposure to light
10 activates the transcription of a number of genes in the common
bread mold, *Neurospora*. Several of these genes produce products
which are essential enzymes in the synthetic pathway of
carotenoids. A number of distinct genetic mutants in this
pathway (designated Albino-1, -2 and -3 or al-1, al-2 and al-3)
15 were described in a paper by Harding, R.W. et al. Plant Physiol
(1981) 68:745-749. The genes associated with these mutations
are light-regulated, and the genes associated with all three of
the al-1, al-2 and al-3 mutants have been cloned (Nelson, M.A.
et al. Mol Cell Biol (1989) 9:1271-1276; Schmidhauser, T.J. et
20 al. Mol Cell Biol (1990) 10:5064-5070).

The al-3 gene controls the production of geranyl geranyl
pyrophosphate synthetase. Geranyl geranyl pyrophosphate is
precursor for carotenoids and xanthophylls. Previous work has
shown that the synthesis of this intermediate is controlled at a
25 transcriptional level and that a 30-minute pulse of light causes
a 15-45 fold coordinated increase in the transcription of the
three genes in the carotenoid biosynthetic pathway. The
conditions for activating the al-3 promoter using light are
known, for example, as described by Baima, S. et al. J Photochem
30 Photobiol B: Biol (1992) 15:239-251.

Because it is often convenient to regulate the expression
of genes encoding heterologous proteins in recombinant
production systems, placing the relevant coding sequence under
control of a light-regulated promoter would offer a simple and
35 effective way to control expression and adapt its timing to the
physiological status of the host. Heretofore, there has been no

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utilization of this means of regulation in expression systems associated with filamentous fungal hosts. The present invention provides such a regulated system.

Disclosure of the Invention

5 The invention provides expression systems for heterologous proteins in filamentous fungi which can be regulated by the presence or absence of light. These expression systems place the nucleotide sequence encoding a heterologous protein under control of the promoter associated with the al-1, al-2 or al-3
10 gene. Advantageously, the expression system is contained on a nucleic acid molecule that further comprises a nucleotide sequence which, in the context of the fungal host used, will provide a selectable marker function.

 Accordingly, in one aspect, the invention is directed to a
15 nucleic acid molecule which comprises a first nucleotide sequence encoding a heterologous protein operably linked to the al-1, al-2 or al-3 promoter and optionally further comprises a second nucleotide sequence that provides for selectable marker means in the fungus chosen.

20 In other aspects, the invention is directed to filamentous fungi modified to contain the nucleic acid molecules of the invention and to methods to produce heterologous proteins by culturing these fungi.

Brief Description of the Drawings

25 Figure 1 shows the nucleotide sequence of the al-3 promoter insert used to construct the plasmids of the invention.

 Figure 2 shows the effect of light on growth of *N. crassa* cultures transformed with various plasmids containing the open reading frame (ORF) of the *mtr* gene under control of the al-3
30 promoter.

 Figure 3 is an alternative form of the data in Figure 2 wherein the growth in darkness has been normalized.

 Figure 4 shows the data obtained in an experiment similar to that depicted in Figure 2 with a fresh culture of the
35 untransformed strain.

 Figure 5 is an alternative form of the data in Figure 4

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wherein the growth in darkness has been normalized.

Figure 6 is a diagram showing the construction of pLRC.

Figure 7 shows the nucleotide sequence of the chymosin open reading frame.

5 Modes of Carrying Out the Invention

The invention provides expression systems useful when filamentous fungi are used as recombinant hosts. "Filamentous fungi" refers to fungi that can form a mycelium through a mass of branching, interlocking filaments. Although these branches
10 may be interrupted by cross-walls, the passage of cytoplasm between compartments is possible. Both sexual and asexual reproduction occur in these fungi. In asexual reproduction, spores known as "conidia" are borne externally at the tips of budding projections formed at various locations along the
15 filaments. The families represented by filamentous fungi include *Phycomycetes*, *Ascomycetes*, *Basidiomycetes* and *Deuteromycetes*. The most popular family is the *Ascomycetes* which includes the genera *Neurospora*, *Aspergillus* and *Penicillium*. Particularly preferred hosts include *A. nidulans*,
20 *A. niger* and *N. crassa*, especially *N. crassa*.

The Light-Regulated Expression System

The nucleic acid molecules of the invention comprise an expression system utilizing the al-1, al-2 or al-3 promoter operably linked to a nucleotide sequence encoding a heterologous
25 protein. The expression system is typically supplied along with a selectable marker means. The selectable marker means may reside on an additional vector or may be included in the nucleic acid molecule which contains the expression system. The nature of the selectable marker means will depend on the nature of the
30 host and the culture conditions.

The expression system itself will comprise the required promoter as well as, if desired, other features which help regulate expression such as enhancers, terminator sequences, polyadenylation sequences and the like as is understood in the
35 art. Means for constructing such expression systems from nucleic acid of known sequence is well understood and employs

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conventional techniques such as those set forth in Maniatis et al., Molecular Cloning: A Laboratory Manual (1982); Gover, D.N. et al. DNA Cloning: A Practical Approach (1985) Vols. I and II and other standard texts commonly available to practitioners.

5 In general, the al-1, al-2 or al-3 promoter is placed in operable linkage with a desired coding sequence for expression. Suitable coding sequences are those for a variety of proteins including enzymes such as urokinase, tissue plasminogen activator, or collagenase; hormones such as human, bovine or
10 chicken growth hormones, gonadotropins such as follicle stimulating hormone, luteinizing hormone, thyroid stimulating hormone or human chorionic gonadotropin, insulin, including human, porcine or bovine insulin, ACTH, prolactin and the like; peptides that mediate physiological functions such as atrial
15 natriuretic peptide, erythropoietin, bradykinin and brain natriuretic peptide; cytokines such as the interleukins, colony stimulating factors; immunoglobulins and fragments thereof; certain toxins, such as ricin or diphtheria toxin; enzymes of industrial importance such as proteases, oxidases, peroxidases
20 and the like; receptor proteins such as thrombin receptor, calcium receptors and the like; nutritional and structural proteins such as sulfur-rich proteins or collagen; growth factors such as TGF α and TGF β , PDGF, EGF, IGF and FGF; and, in general, any protein whose recombinant production is desired and
25 for which a coding nucleotide sequence can be obtained.

Some of the desired proteins may be heterodimers; in this instance, multiple expression systems involving the promoters of the invention will be used. Both subunits of the heterodimer may be produced by a single vector, or multiple vectors may be
30 used for transformation.

The constructs may be designed, as is generally recognized, to provide a signal sequence for secretion of the desired protein into the culture medium, or the protein may be produced intracellularly. If desired, the protein may be designed as a
35 part of a fusion protein, which can later be cleaved to yield the desired product. Methods for operably linking the promoters of the invention to the desired coding sequence in the above-mentioned environments are generally understood in the art.

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Selectable Markers

As the filamentous fungal hosts need to be modified to accommodate expression systems of the invention, it is desirable to include a selectable marker in the transformation protocol.

5 The selectable marker can be placed on the same vector as that which contains the expression system of the invention, or may be part of an additional vector used to cotransform the host. The appropriate choice of selectable marker will depend on the nature of the host. A straightforward choice might be, for
10 example, an expression system which produces an enzyme responsible for an antibiotic resistance against an antibiotic to which the host is susceptible, such as benomyl. Alternatively, if a host is chosen with, for example, a nutritional deficiency, the wild-type gene can be used to
15 replace the deficiency. To do so, however, requires a suitable mutant.

Although mutants can be prepared generally, the better studied species of filamentous fungi are preferred because numerous mutants are readily available which make design of
20 transformation vectors containing means for selection more diverse. For example, one very simple method for selection utilizes a mutant host with a requirement for a particular nutrient where the selectable marker means is provided by replacing the defective gene which accounts for this nutritional
25 requirement. As an illustration, if a mutant unable to grow in the absence of histidine is used as a host, successful transformants can be selected using as a "marker" nucleic acid containing the wild type of the gene that is defective in the mutant and growing the transformed cells on minimal media. Only
30 the successful transformants will be able to grow in the absence of histidine. Similar mutations which result in dependence on the presence of other amino acids or other nutrients in the media are also known. In *N. crassa*, for instance, known mutants include mutants which have specific nutritional requirements.
35 Examples of useful nutrient requirements and the relevant mutants include:

(1) amino acids such as histidine (his-1 through -7 mutants), proline (aga mutants), arginine (arg-11 mutants),

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citrulline (arg-11 mutants), asparagine (asn mutants), choline (chol-1 and chol-2 mutants), cysteine (cys-1 mutants), glutamine (gln-1 mutants), leucine (leu-1 through -4), lysine (lys-2, -4 and -5), methionine (mac mutants and met-6, -9 and -10 mutants),
5 and threonine (thr-2 and -3 mutants);

(2) mixtures of aromatic amino acids, such as a mixture of p-aminobenzoic acid, tyrosine, tryptophan, and phenylalanine (required by all aro strains except aro-6, aro-7 and aro-8), a mixture of tryptophan and phenylalanine (required
10 for aro-6 mutants), a mixture of isoleucine and valine (required for ilv-1, -2 and -3), and a mixture of phenylalanine and tyrosine (required for pt mutants);

(3) vitamins such as pantothenic acid (pan-1 mutants) and thiamine (thi-2 and thi-4 mutants);

15 (4) purine bases such as adenine (ad-2 through ad-4 and ad-8 mutants), hypoxanthine (ad-2 and ad-3 mutants), inosine, and guanine or guanosine (gua-1 or -2 mutants);

(5) pyrimidine bases such as uracil (pyr-1 through pyr-6);

20 (6) saturated fatty acids (cel mutants) or unsaturated fatty acids such as C₁₆ or C₁₈ fatty acids having a double bond in the cis conformation at either the 9- or 11-position, fatty acids with a double bond in the trans configuration at the 9-position, and fatty acids with multiple
25 cis double bonds interrupted by methylene bridges (ufa-1 and -2);

(7) physiologically important ions such as potassium (trk);

(8) sugar alcohols such as inositol (acu mutants and inl mutants) and glycerol; and

30 (9) other organic entities such as acetate (ace mutants), α -ketoglutarate, succinate, malate, formate or formaldehyde (for mutants), p-aminobenzoic acid (pab-1, -2 and -3 mutants), and sulfonamide (sfo mutants at 35°C).

One specific example based on a nutritional
35 requirement is the Arg B⁺ gene coding for the enzyme ornithine transcarbamylase. This enzyme is present in wild type *A. niger*. Mutants lacking this enzyme (Arg B⁻ strains) can be prepared by usual non-specific techniques, such as treatment with

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ultraviolet radiation, followed by screening based on an inability to grow on minimal medium, coupled with an ability to grow on a medium containing arginine. Fungi containing this genome will grow on minimal medium if they also include an ArgB+ nucleus.

Other selectable markers confer resistance to toxins or to other adverse culture conditions such as high temperature. Specific examples of noxious chemicals that can exert a toxic effect include acriflavine (resistance conferred by *acr* generally, with the presence of the *shg* gene being required for resistance by *acr-4* and *acr-6*); 3-amino-1,2,4-triazole (resistance conferred by *acr-2*, *atr-1*, *cpc*, *leu-1* or *leu-2*); dyes such as malachite green (resistance conferred by *acr-3*); caffeine (resistance conferred by *caf-1*); purine analogs (resistance to 8-azaadenine and 2,6-diaminopurine conferred by *aza-1*; resistance to 8-azaadenine and 8-azaguanine conferred by *aza-2*; resistance to 8-azaguanine and 6-mercaptopurine conferred by *aza-3*; resistance to 6-methylpurine conferred by *mep(3)* and *mep(10)*; cyanide (insensitivity conferred by *cni-1* in the first 24 hours of growth); tetrazolium (resistance conferred by *cya-6* and *cya-7*); cycloheximide (resistance conferred by *cyh-1*, *-2* and *-3*); chromate (resistance conferred by *cys-13*); 2-deoxy-D-glucose (resistance conferred by *dgr*); edeine (resistance conferred by *edr-1* and *-2*); ethionine (resistance conferred by *eth-1*, by *nap* in the presence of p-fluorophenylalanine, and by *oxD* if the ethionine is in the D form); fluoro compounds such as 5-fluorodeoxyuridine, 5-fluorouracil, and 5-fluorouridine (resistance to all three conferred by *fdi-2*; resistance to 5-fluorouracil being conferred by *uc-5* in an ammonia-free minimal medium; resistance to 5-fluorodeoxyuridine and 5-fluorouridine being conferred by *ud-1*), and fluorophenylalanine (resistance conferred by *fpr-1* through *-6* under certain conditions); 8-azaadenine (resistance conferred by *mts*); methyl methane sulfonate (insensitive or marginally sensitive for *upr-1*); surface-active agents such as dequalinium chloride, cetyltrimethyl ammonium bromide, and benzalkonium chloride (resistance conferred by *sur-1*); and metal ions such as vanadate (resistance conferred by *van*).

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Also useful are genes conferring resistance to extremes in various environmental conditions such as a high or low temperature, the lack of oxygen (resistance conferred by *an*), constant light (resistance conferred by *lis-1*, *-2* and *-3*) or the
5 absence of light, UV radiation, ionizing radiation, and high or low osmotic pressures.

Many strains may be obtained from the Fungal Genetics Stock Center (FGSC). Other useful strains can be prepared using known techniques. For example, a strain having the characteristics of
10 *A. niger* (ATCC 46951) can be prepared by mutagenizing with UV light to form an isolate that requires ornithine or arginine for growth in a defined minimal media. This strain, which lacks ornithine carbamoyl transferase, has been called *arg B* (350(-)52). Media for growing *A. niger* or *A. nidulans* are described
15 by Cove, Biochim Biophys Acta (1966) 113:51-56.

However, other selectable marker systems can also be used, such as the inclusion of genes which confer resistance to toxic substances or other detrimental culturing conditions. For
example, genes encoding proteins which confer resistance to
20 antibiotics can be used where selection is conducted on media containing the antibiotic. In the case of filamentous fungi, such antibiotics include benomyl.

More complex systems of selection involve inactivation of endogenous genes that confer susceptibility to poisons. For
25 example, illustrated herein is selection by inactivation of the endogenous *mtr* locus which confers susceptibility to *p*-fluorophenylalanine (*pfpa*) or 4-methyltryptophan (4-MT). Inactivation occurs by homologous recombination into the *mtr* locus so as to disrupt its function. Homologous recombination in
30 filamentous fungi is well established. See, e.g. Asch, D.K. et al. Mol Gen Genet (1990) 221:37-43; Asch, D.K. et al. Genetics (1992) 130:737-748. An alternative approach would utilize inactivation of the endogenous *pmb* locus which confers sensitivity to canavanine. For these methods of selection, only
35 hosts homokaryotic ~~for~~ transformation are selected. Since filamentous fungi can harbor a multiplicity of nuclei in what is essentially a single cytoplasm, if any one of the nuclei contains an intact gene conferring sensitivity, the fungus will

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fail to survive in the presence of the toxin.

Thus, in one particularly preferred embodiment, the expression system of the invention may be inserted into a vector which accomplishes homologous recombination into the *mtr* locus.

5 In this case, preferably, regions of the *mtr* gene which are insufficient to provide the product of the *mtr* gene, but sufficient to promote homologous recombination are contiguous with the expression system of the invention or with the nucleotide sequence encoding the desired heterologous protein.

10 Vectors which provide such *mtr* sequences and means for employing these vectors to effect homologous recombination and thus resistance to pfpa are described in copending application 08/105,448 filed 12 August 1993 the contents of which are now published in PCT application WO 93/25663 and incorporated herein

15 by reference. Described in this application is a vector, there designated pXpress, which provides insertion sites for either the coding sequence for the desired protein or the expression system for the protein or both into the *mtr* gene such that homologous recombination of the vector with the endogenous *mtr*

20 confers resistance to this toxin.

Systems of selection which are more subtle in their design may also be used. For example, a double mutant which contains a nonfunctional *mtr* gene (*mtr'*) and which also is unable to synthesize tryptophan can be selected for transformation by

25 using the functional *mtr* gene as a marker and culturing the transformants in a medium containing high levels of arginine along with tryptophan. Since the mutant requires tryptophan for growth, it must transport tryptophan from the medium. There are two possible gene products which permit transport of tryptophan:

30 the *mtr* gene product and the *pmg* gene product. However, arginine competes for *pmg*, so the tryptophan can be transported effectively only if a functional *mtr* gene is present. Thus, only successful transformants will grow on minimal medium containing a tryptophan supplement along with high levels of

35 arginine. Thus, employing this strategy, the *mtr* gene sequences can also provide for selection by encoding a product which restores a nutritional deficiency under specified conditions. The vectors described in the above-referenced

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copending application 08/105,448 and PCT publication WO 93/25663 can also be manipulated to provide the mtr product for such selection procedures.

Transformation and Culture

5 Standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, Methods Enzymol (1971) 17A:79-143. Standard procedures are generally used for the maintenance
10 of strains and the preparation of conidia. Mycelia are typically grown in liquid cultures for about 14 hours (25°C), as described in Lambowitz et al., J Cell Biol (1979) 82:17-31. Host strains can generally be grown in either Vogel's or Fries minimal medium supplemented with the appropriate nutrient(s),
15 such as, for example, histidine; arginine; phe, tyr, and/or trp (each about 80 µg per ml); p-aminobenzoic acid (about 2 µg per ml); and inositol (about 0.2 mg per ml).

 Since the promoters of the invention are regulated by light, the cultures can be grown under conditions of darkness
20 until expression is desired. The expression systems can be activated by illuminating the cultures using light radiation which contains blue wavelengths suitable for such activation, as described by Harding, R.W. and Turner, R.V., Plant Physiol (1981) 68:745-749 cited above.

25 When expression has been activated and the desired protein produced, the protein may be recovered from the culture using techniques generally recognized in the art. If the protein is produced intracellularly, the cells are lysed and the lysate subjected to suitable separation and purification procedures.
30 If the protein is secreted into the medium, the medium can be removed and the secreted protein purified using conventional techniques such as size exclusion, ion exchange chromatography, reverse phase chromatography, differential centrifugation, and the like. Suitable protocols will depend on the nature of the
35 protein product.

 If the protein is provided as a fusion protein, it is preferable that a protease site be inserted into the sequence so

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that liberation of the mature protein is a simple matter of treatment with the appropriate protease.

Examples

The following examples are intended to illustrate but not
5 to limit the invention.

Example 1

Construction of an Expression System and Expression of a Model Gene

As a model system, the open reading frame (ORF) of the *mtr*
10 *N. crassa* gene was placed under the control of the *al-3* promoter
in plasmids designated pLRN and pALN and transformed into
N. crassa under conditions which permit the *mtr* gene to provide,
itself, a selectable marker function. Transformation of
N. crassa with these vectors resulted in superior growth in the
15 presence of light and in enhanced production of mRNA encoding
the *mtr* gene product. The following describes the construction
and expression.

N. crassa strain 82-59 (*trp-2*, *mtr*, *cot-1*, *ylo-a*) obtained
from D.D. Stadler, U. of Washington at Seattle was used as host.
20 This strain requires tryptophan for growth and has a
nonrevertable *mtr* phenotype containing a small deletion at site
1536 in the *mtr* ORF. Thus, this mutant cannot grow in the
absence of tryptophan, and cannot grow even in the presence of
tryptophan when excess arginine is present unless *mtr*
25 functionality is restored.

The host strain was maintained on conidia growth slants in
medium containing 1 X Vogel's Medium, 2% sucrose, 0.05 mg/ml
tryptophan.

Two plasmids were constructed placing the *mtr* ORF under
30 control of the *al-3* promoter. pLRN was constructed by ligating
a 1.2 kb PstI/SalI fragment containing the *al-3* promoter
retrieved from 1.0 μ g of an M13mp18 vector provided by Guiseppe
Macino, University of Rome and gel purified using Gene Clean II
Kit (Bio 1011), into a PstI/SalI digest of 1 μ g plasmid pN846
35 described in detail in U.S. Serial No. 08/105,448 filed
12 August 1993 and PCT WO 93/25663 cited above. The complete

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sequence of the al-3 promoter-containing insert is shown in Figure 1. Dr. Macino has identified the region responsible for photoinduction to reside between positions -55 to -225 from the transcription start site. The digested materials were incubated
5 overnight at 15°C with 400 U of T4 ligase. The ligation mixture was transformed into *E. coli* DH5A made competent with treatment by calcium chloride and transformed colonies were screened for the insert using restriction digest analysis.

The linearized vector obtained by PstI/SalI digest of pN846
10 provides a portion of the *mtr* promoter, the entire ORF and terminator sequences. Ligation of this fragment with the 1.2 kb al-3 promoter fragment places the *mtr* ORF under control of the al-3 promoter. The resulting vector was designated pLRN (for light regulated neutral).

15 An analogous vector was also prepared placing the al-3 promoter 5' of the complete *mtr* insert in pN846, thus effectively 5' of the native *mtr* promoter. In this plasmid, the complete *mtr* promoter is located between SalI sites at positions 1336-1660, i.e., between the al-3 promoter and the *mtr* ORF.
20 This plasmid was designated pALN.

Spheroplasts of strain 82-59 were prepared using the methods described by Vollmer, S.J. et al. Proc Natl Acad Sci USA (1986) 83:4869-4873. Transformation was conducted as described by Stuart, W.D. et al. Genome (1988) 30:188-203.

25 Selection was conducted using bottom plates made with Vogel's Media and FIGS (0.5% fructose, 0.2% inositol, 0.5% glucose, 20% sorbose) as the carbon source, and in the presence of 0.1 mg/ml arginine and 0.01 mg/ml tryptophan. The cultures were incubated in a light-sealed 28°C incubator. Regulation of
30 light conditions was accomplished with a Micronta Programmable Timer (Radio Shack Catalog No. 63-864) to cycle the light every two hours. Colonies were scored after two days and transferred to high selection conidial growth media on slants. In the presence of sorbose, *N. crassa* takes on a colonial phenotype
35 which permits isolation of a single transformed cell. Minimal top agar (1M sorbitol, 1X Vogel's salts and 2.8% bacto agar) was also supplemented with 0.1 mg/ml arginine.

The transformation frequencies per 4.6×10^6 cells were

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approximately 20 and 70 for pLRN and pALN respectively.

Homokaryotic transformants were obtained by transferring conidia to slants containing supplements of 0.05 mg/ml pfpa and 0.05 mg/ml anthranilic acid. (Anthranilic acid was added to
5 obviate the trp-2 mutation; addition of tryptophan would have resulted in competition with pfpa for the transport system.) These were checked for sensitivity to pfpa in the presence of light. A conidia suspension of colonies that showed light sensitivity were plated on the high selection medium described
10 above which includes 0.1 mg/ml arg and 0.01 mg/ml trp in order to isolate homokaryotic transformed cells. Plating and restreaking was repeated until 100% sensitivity to pfpa in the light was obtained and successful growth on high selection media was observed. This ensures isolation of homokaryons.

15 The resulting homokaryons were examined for the effect of light on conidial growth. Liquid Vogel's minimal medium (1X Vogel's salts and 2% sucrose) supplemented with 0.015 mg/ml pfpa and 0.05 mg/ml anthranilic acid was inoculated with either 30,000 or 60,000 conidia and cultured in the light and dark at
20 28°C for 5 days. The fungi were then harvested by filtration and the dry weight of cell mass measured. Wild type strain 74a was used as a control.

The results are shown in Figure 2. An alternate form of these results where growth in light is normalized to 1 mg is
25 shown in Figure 3. As expected, wild type strain 74a is always sensitive to pfpa and thus does not grow. The untransformed strain 82-59 is relatively tolerant to pfpa since it has a nonrevertable *mtr* phenotype. The selected homokaryons show a greater differential between light and dark growth since in the
30 dark, their *mtr* phenotype is retained, while in the light the *mtr* gene is expressed rendering them susceptible to pfpa poisoning. Thus, as indicated, a number of the transformed strains showed a greater light sensitivity as compared to the parental strain 82-59. This experiment was repeated with a
35 fresh culture of the untransformed strain 82-59 to obtain similar results as shown in Figure 4; the normalized results obtained in Figure 4 are shown in Figure 5.

The transformed pLRN 13-1 was selected for further study

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since it showed the greatest differential between light and dark conditions.

Messenger RNA from pLRN13-1 and 82-59 was subjected to Northern Blot analysis and probed with 1050bp of the *mtr* ORF.

5 The results for pLRN13-1 showed a high level of production of mRNA hybridizing with the probe in the presence of light, but very little in the dark. Strain 82-59 did not show production of *mtr* mRNA under either condition.

10 Thus, using the *mtr* coding sequence as a model system, it is demonstrated that al-3 can regulate the expression of this ORF as a function of the presence of light.

Example 2

Expression of a Mammalian Gene

15 The mammalian gene encoding chymosin was placed in operable linkage with the al-3 promoter as shown in Figure 6.

A 1 μ g sample of the 6.6 kb plasmid pLRN was digested with SalI and purified. SalI cleaves between the al-3 promoter and the *mtr* ORF. The chymosin gene was released with XhoI and SalI from a cDNA chymosin clone obtained from Berlex Biosciences and
20 gel purified. The DNA sequence encoding chymosin is shown in Figure 7. The ORF extends from the ATG at position 72 to the TGA at position 1218. The chymosin ORF was ligated in proper orientation in the cleaved vector providing the intermediate pCLRn containing the chymosin ORF immediately downstream and in
25 operable linkage to the al-3 promoter and immediately upstream of the *mtr* ORF. Proper insertion was verified by restriction analysis. pCLRn was digested with HincII and recircularized with 400 U of T4 ligase. This deletes approximately 1.1 kb of the *mtr* ORF. The resulting 6.6 kb plasmid was designated pLRC.
30 pLRC thus contains an expression system for chymosin under control of the al-3 promoter contiguous with a nonfunctional portion of the *mtr* ORF sufficient to effect homologous recombination.

The host spheroplasts were obtained from strain His-3
35 TM428A, FGSC4438. This mutant will grow only in the presence of histidine and a plasmid capable of restoring histidine biosynthesis, pNH60, containing the *his* gene and obtained from

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FGSC, was used for cotransformation selection. Transformation was conducted using 10 μ g pLRC DNA and 2 μ g of pNH60 DNA as described above. The transformants were selected as described above using bottom plates and top agar made with Vogel's Media and FIGS in the absence of amino acid supplements. This resulted in isolation of 44 transformants. An inoculum of each into 1X Vogel's with 2% sucrose liquid medium was made and the cultures were incubated at 28°C for 7 days in constant 2-hour light cycling.

10 For 41 of the transformants, 1 ml of liquid medium was blotted onto MSI charged nylon membrane using the Minifold-II Slot Blot System. The filter was treated with TBS (20 mM tris-HCl, 500 mM NaCl) and 10 mM EDTA, pH 8.0 and incubated at 70°C for 30 minutes to inactivate endogenous alkaline phosphatase.

15 The filter was rinsed in TBS and blocked using 10% nonfat dry milk in TBS for 1 hour at 37°C. The block was drained and the primary antibody (rabbit anti-prochymosin) at a 1:5000 dilution in 5% nonfat milk TBS was added and the mixture incubated at 37°C for 1 hour. The filter was drained and washed in 0.05% TWEEN TBS (TTBS) 2 times (5 minutes). The secondary antibody (goat antirabbit alkaline phosphatase conjugate) was added at a final concentration of 0.24 μ g/ml and incubated at 37°C for 1 hour. The filter was washed and the color substrate added according to the instructions of the manufacturer (Boehringer Mannheim Genius Kit) (45 μ l of 75 mg/ml Nitroblue tetrazolium salt, 35 μ l of 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate diluted in 10 ml of 100 mM tris-HCl, 100 mM NaCl and 50 mM MgCl₂, pH 9.5).

The results showed that 27 of the 41 transformants produced 30 chymosin. However, when 6 of these producers were selected and assayed by SDS PAGE (Western Blot), no chymosin was detected. The experiment was repeated using 3 of the selected strains and culturing 9 days at 28°C in light cycled as above. 450 μ l of liquid media were treated with various concentrations of 0.5-2 M 35 KCl to release proteins from cell walls and analyzed by the Slot Blot method described above. In addition, several cultures were treated with 50 μ g/ml lysozyme to cause apical swelling and lysis of the mycelia. Slot Blot analysis of each of these

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cultures treated of showed higher detection of chymosin.

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Claims

1. A nucleic acid molecule for expression of a first nucleotide sequence encoding a heterologous protein in a filamentous fungus, which nucleic acid molecule comprises said
5 first coding sequence operably linked to the al-1, al-2 or al-3 promoter and optionally further comprises a second nucleotide sequence that provides for a selectable marker means in said fungus.

2. The nucleic acid molecule of claim 1 wherein said
10 second nucleotide sequence is present and promotes homologous recombination of said nucleic acid molecule with the fungus chromosome so as to alter a region of said chromosome thus providing a selectable marker means.

3. The nucleic acid molecule of claim 2 wherein said
15 second sequence is contiguous with said first sequence.

4. The nucleic acid molecule of claim 2 wherein said second sequence corresponds to at least a portion of the *mtr* locus.

5. The nucleic acid molecule of claim 1 wherein said
20 second sequence is present and encodes a protein that confers a selectable characteristic on said fungus.

6. The nucleic acid molecule of claim 5 wherein said selectable characteristic is conferred by the product of the *mtr* gene.

25 7. The nucleic acid molecule of claim 1 wherein said first sequence encodes chymosin or relaxin.

8. A filamentous fungus modified to contain the nucleic acid molecule of claim 1.

9. The fungus of claim 8 which is a species of

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Neurospora.

10. A method to produce proteins heterologous to a filamentous fungus, which method comprises culturing the host fungus of claim 8 under conditions wherein said heterologous protein is produced; and
5 recovering the heterologous protein from the culture.

DNA SEQUENCE 1237 b.p. AAGCTTGCATGC ... GAGCTCGAATTC LINEAR

1	AAGCTTGCA	10	1	20	1	30	1	40	1	50	1	60
61	CGACAGACGT	GCCTG	CAGCT	GCCTG	CAGCT	GCCTG	CAGCT	GCCTG	CAGCT	GCCTG	CAGCT	GCCTG
121	TAAAGTGCC	AGACG	TCC	AGACG	TCC	AGACG	TCC	AGACG	TCC	AGACG	TCC	AGACG
181	AGTGACTGA	ACTCG	ACGT	ACTCG	ACGT	ACTCG	ACGT	ACTCG	ACGT	ACTCG	ACGT	ACTCG
241	CCACAACAGC	CGACCT	CGTT	CGACCT	CGTT	CGACCT	CGTT	CGACCT	CGTT	CGACCT	CGTT	CGACCT
301	CGAGCCCCCT	ACCA	GCAACA	ACCA	GCAACA	ACCA	GCAACA	ACCA	GCAACA	ACCA	GCAACA	ACCA
361	TCTGAAGCCC	TGACA	AGACA	TGACA	AGACA	TGACA	AGACA	TGACA	AGACA	TGACA	AGACA	TGACA
421	AGGTGAGAAA	CCCTC	CCCA	CCCTC	CCCA	CCCTC	CCCA	CCCTC	CCCA	CCCTC	CCCA	CCCTC
481	CGACAAAGAG	CGCG	CGAG	CGCG	CGAG	CGCG	CGAG	CGCG	CGAG	CGCG	CGAG	CGCG
541	CCCCCTGTGA	GAGCT	CCCCG	GAGCT	CCCCG	GAGCT	CCCCG	GAGCT	CCCCG	GAGCT	CCCCG	GAGCT
601	CGGGGTTTCA	TCTTG	CAAA	TCTTG	CAAA	TCTTG	CAAA	TCTTG	CAAA	TCTTG	CAAA	TCTTG
661	AATCACCTTG	CATCC	ACTG	CATCC	ACTG	CATCC	ACTG	CATCC	ACTG	CATCC	ACTG	CATCC
721	GCCTACGGC	AGCTG	ATCC	AGCTG	ATCC	AGCTG	ATCC	AGCTG	ATCC	AGCTG	ATCC	AGCTG
781	ATCCAGTGAC	GATGG	CTGGC	GATGG	CTGGC	GATGG	CTGGC	GATGG	CTGGC	GATGG	CTGGC	GATGG
841	ACCTAGCAGA	AAGAG	GCCTT	AAGAG	GCCTT	AAGAG	GCCTT	AAGAG	GCCTT	AAGAG	GCCTT	AAGAG
901	TAGATCTCTT	GGCCT	TGTT	GGCCT	TGTT	GGCCT	TGTT	GGCCT	TGTT	GGCCT	TGTT	GGCCT
961	AAAGTGAGGT	CGATT	GCTGT	CGATT	GCTGT	CGATT	GCTGT	CGATT	GCTGT	CGATT	GCTGT	CGATT
1021	CGTGCGGGTA	TGAA	TATTG	TGAA	TATTG	TGAA	TATTG	TGAA	TATTG	TGAA	TATTG	TGAA
1081	ACGTCAAGTA	ATTAA	AGAA	ATTAA	AGAA	ATTAA	AGAA	ATTAA	AGAA	ATTAA	AGAA	ATTAA
1141	GGGGTTAGCA	TCCTC	TACAG	TCCTC	TACAG	TCCTC	TACAG	TCCTC	TACAG	TCCTC	TACAG	TCCTC
1201	AGGATCCGGC	GGCCCC	CCCGG	GGCCCC	CCCGG	GGCCCC	CCCGG	GGCCCC	CCCGG	GGCCCC	CCCGG	GGCCCC

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□ GROWTH IN THE LIGHT (mg)
 ■ GROWTH IN THE DARK (mg)

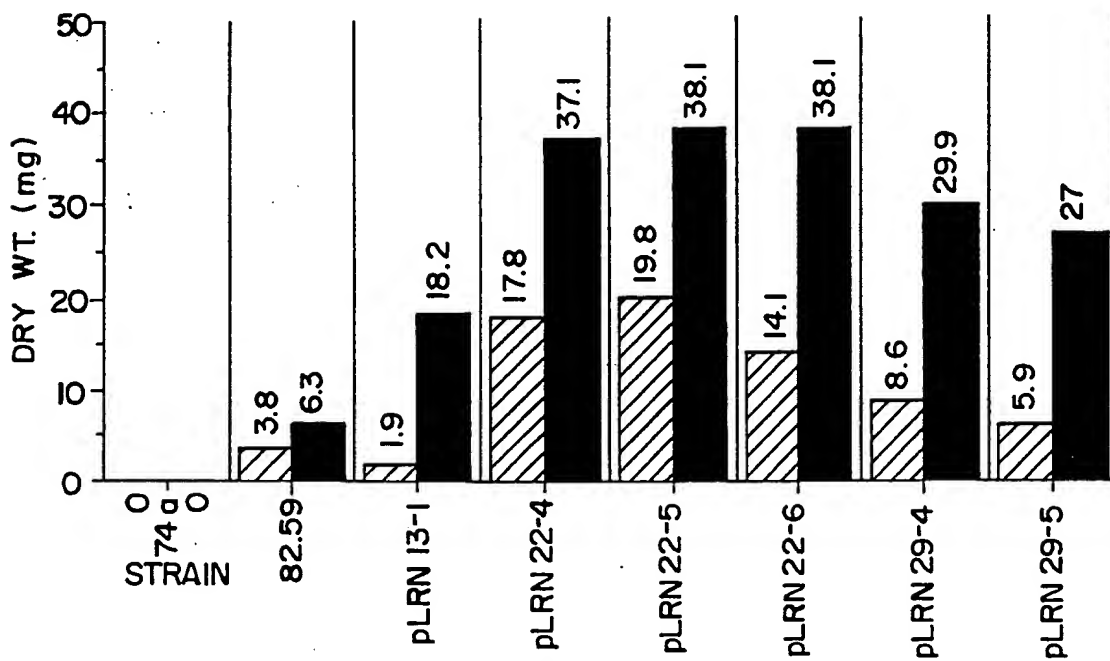


FIG. 2

□ NORMALIZED GROWTH (LIGHT)
 ■ NORMALIZED GROWTH (DARK)

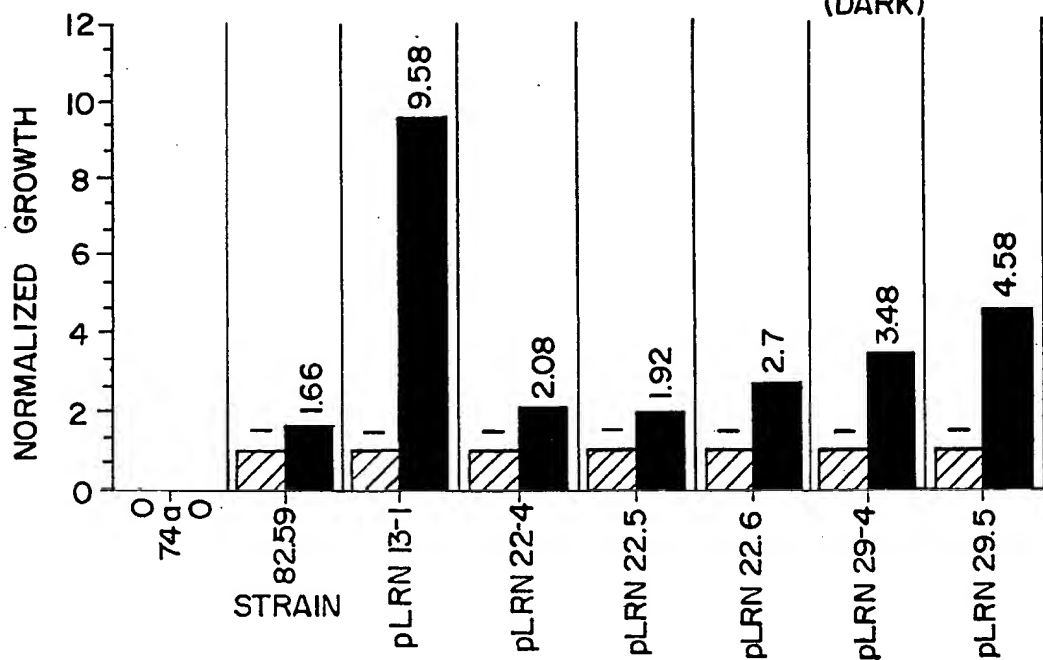


FIG. 3

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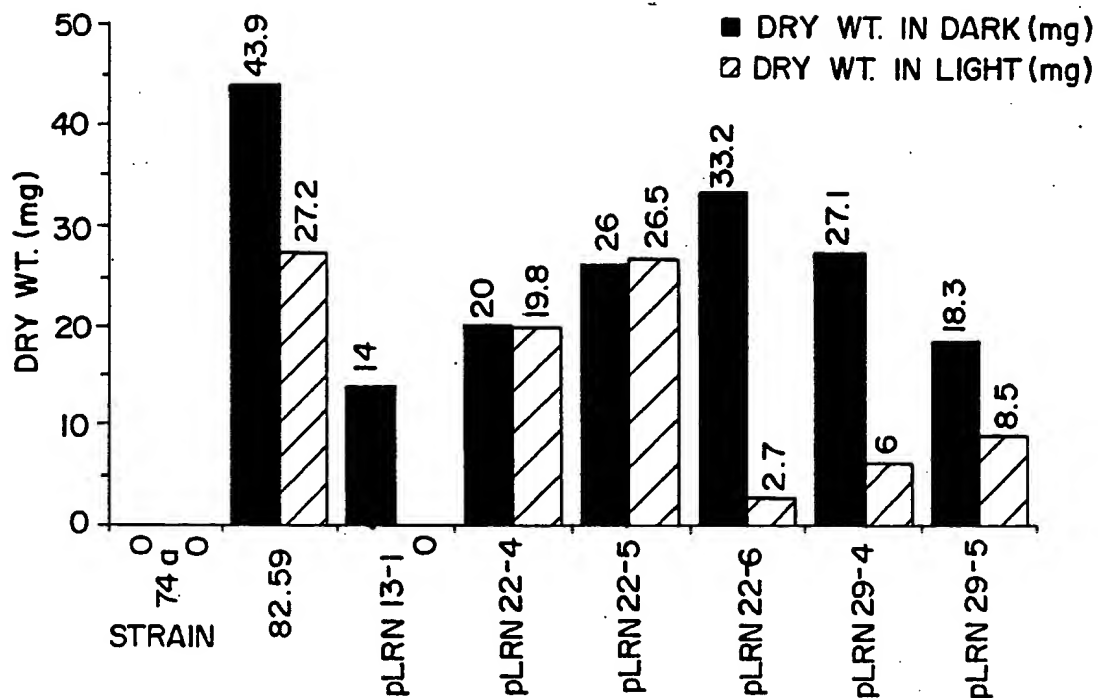


FIG.4

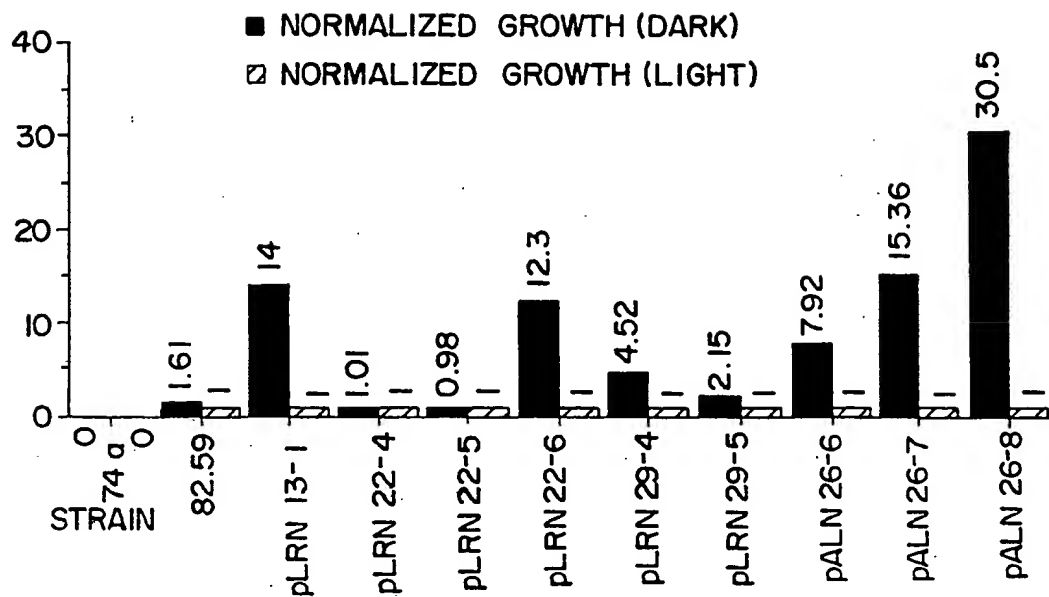


FIG.5

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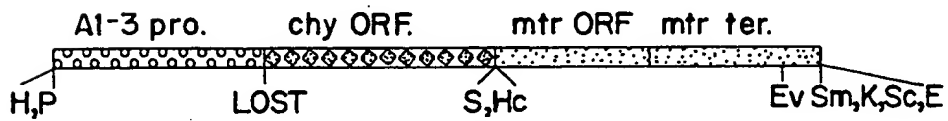
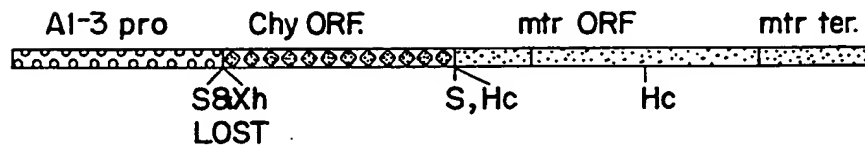
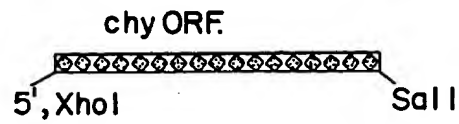
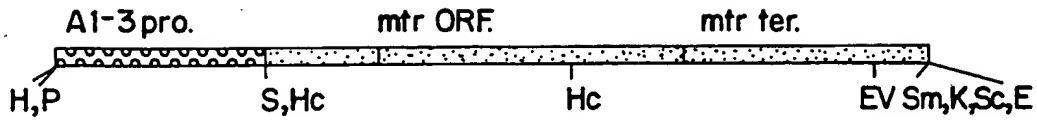


FIG.6

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PBC18XH ->LIST

DNA SEQUENCE		1240 b.p. gaattcgagctc ... gcagccaaagctt		LINEAR	
1	gaattcgagc	1	10	1	60
61	ccagatccaa	21	20	51	60
121	ctgagatcac	31	20	61	60
181	atgggcttct	41	20	71	60
241	gcttcgggga	51	20	81	60
301	agatctacct	61	20	91	60
361	acttctgggt	71	20	101	60
421	acccgagaaa	81	20	111	60
481	caggcagcat	91	20	121	60
541	tccaggagac	101	20	131	60
601	tcgacgggat	111	20	141	60
661	ttgacaacat	121	20	151	60
721	ggaatggcca	131	20	161	60
781	ccctgcactg	141	20	171	60
841	ccatcagcgg	151	20	181	60
901	cctccaagct	161	20	191	60
961	cacagaacca	171	20	201	60
1021	tggctcttga	181	20	211	60
1081	accagggttt	191	20	221	60
1141	gggatgtttt	201	20	231	60
1201	tggccaaaagc	211	20	241	60
		221	20	251	60
		231	20	261	60
		241	20	271	60
		251	20	281	60
		261	20	291	60
		271	20	301	60
		281	20	311	60
		291	20	321	60
		301	20	331	60
		311	20	341	60
		321	20	351	60
		331	20	361	60
		341	20	371	60
		351	20	381	60
		361	20	391	60
		371	20	401	60
		381	20	411	60
		391	20	421	60
		401	20	431	60
		411	20	441	60
		421	20	451	60
		431	20	461	60
		441	20	471	60
		451	20	481	60
		461	20	491	60
		471	20	501	60
		481	20	511	60
		491	20	521	60
		501	20	531	60
		511	20	541	60
		521	20	551	60
		531	20	561	60
		541	20	571	60
		551	20	581	60
		561	20	591	60
		571	20	601	60
		581	20	611	60
		591	20	621	60
		601	20	631	60
		611	20	641	60
		621	20	651	60
		631	20	661	60
		641	20	671	60
		651	20	681	60
		661	20	691	60
		671	20	701	60
		681	20	711	60
		691	20	721	60
		701	20	731	60
		711	20	741	60
		721	20	751	60
		731	20	761	60
		741	20	771	60
		751	20	781	60
		761	20	791	60
		771	20	801	60
		781	20	811	60
		791	20	821	60
		801	20	831	60
		811	20	841	60
		821	20	851	60
		831	20	861	60
		841	20	871	60
		851	20	881	60
		861	20	891	60
		871	20	901	60
		881	20	911	60
		891	20	921	60
		901	20	931	60
		911	20	941	60
		921	20	951	60
		931	20	961	60
		941	20	971	60
		951	20	981	60
		961	20	991	60
		971	20	1001	60
		981	20	1011	60
		991	20	1021	60
		1001	20	1031	60
		1011	20	1041	60
		1021	20	1051	60
		1031	20	1061	60
		1041	20	1071	60
		1051	20	1081	60
		1061	20	1091	60
		1071	20	1101	60
		1081	20	1111	60
		1091	20	1121	60
		1101	20	1131	60
		1111	20	1141	60
		1121	20	1151	60
		1131	20	1161	60
		1141	20	1171	60
		1151	20	1181	60
		1161	20	1191	60
		1171	20	1201	60
		1181	20	1211	60
		1191	20	1221	60
		1201	20	1231	60
		1211	20	1241	60

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05716

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/15, 15/11, 15/12, 15/31; C12P 21/02

US CL : 435/254.4, 69.1; 536/23.5, 23.7, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/254.4, 69.1; 536/23.5, 23.7, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

search terms: mtr, chymosin, neurospora, albino, al1, al2, al3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular and Cellular Biology, Volume 10, Number 10, issued October 1990, Schmidhauser et al., "Cloning, sequence, and photoregulation of al-1, a carotenoid biosynthetic gene of Neurospora crassa", pages 5064-5070, see entire article.	1-10
Y	Molecular and Cellular Biology, Volume 9, Number 3, issued March 1989, Nelson et al., "Molecular cloning of a Neurospora crassa carotenoid biosynthetic gene (albino-3) regulated by blue light and the products of the white collar genes", pages 1271-1276, see entire article.	1-10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance		
"E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search

26 JULY 1995

Date of mailing of the international search report

04 AUG 1995

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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05716

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Plant Physiology, Volume 68, issued 1981, Harding et al., "Photoregulation of the carotenoid biosynthetic pathway in albino and white collar mutants of Neurospora crassa", pages 745-749, see entire article.	1-10
Y	WO, A, 93/25663 (GREENWOOD ET AL.) 23 December 1993, see entire document.	4, 6
Y	Gene, Volume 19, issued 1982, Moir et al., "Molecular cloning and characterization of double-stranded cDNA coding for bovine chymosin", pages 127-138, see entire article.	7